A Review on β-alanine Biosynthesis

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Abstract

β-alanine is the only non-proteogenic β-amino acid found in nature and is a precursor for important molecules biosynthesis in many organisms. Specifically, it is a precursor for pantothenate in microorganisms, and a component of the dipeptides anserine and carnosine in mammals. Furthermore, β-alanine plays a role in insect exoskeleton formation and is part of plant environmental stress response. Apart from its natural role in organisms, β-alanine has applications in nutrition, pharmaceuticals, chemical synthesis, and construction fields. Due to its health-promoting benefit, β-alanine is increasingly being used by athletes to augment exercise performance and endurance. Moreover, it is a precursor for the synthesis of pamidronate sodium, balsalazide, and other drugs in the pharmaceutical industry. Additionally, β-alanine has been proposed as an intermediate to industrially relevant nitrogen-containing compounds. Traditionally, β-alanine has been produced through harsh chemical synthesis routes which often yield unwanted by-products. Recently, β-alanine production is moving towards relatively environmental- friendly whole-cell biosynthesis providing an attractive alternative that enables enantiomerically pure compound production. Some bio-based methods, however, are limited by enzyme inhibition, low stoichiometric yield, and expensive production cost. Hence, the purpose of this work is to review the recent significant attempts at addressing the limitations of β-alanine production.

Introduction

β-alanine is the only β-amino acid produced in nature. It does not partake in protein synthesis but is a precursor for many essential molecules in organisms. For example, β-alanine is a precursor to pantothenate in microorganisms, a component of the intracellular pH buffer carnosine in mammals, a part of exoskelaton formation in insects (Wang *et al.*, 2021), and a component of stress response in plants (Pei *et al.*, 2017). Because it is the limiting reactant in carnosine synthesis, β-alanine is increasingly being used as a sports supplement to augment performance and recovery in athletes (Lily and Papandreou, 2014). In addition to its value as a sports supplement, β-alanine is gaining popularity as a precursor molecule to industrially-relevant nitrogen-containing compounds such as acrylamide, acrylonitrile, and poly-β-alanine (nylon-3) (Steunenberg *et al.,* 2013). Conventional chemical routes for β-alanine synthesis utilize harsh conditions and produce undesired by-products (Song *et al.*, 2015), necessitating more environmentally-friendly and efficient β-alanine synthesis methods. Greener β-alanine production methods such as enzymatic approaches entail converting L-aspartate to β-alanine using purified L-aspartate-alpha-decarboxylase (ADC) (Wang *et al.*, 2021). However, the cost of enzyme extraction and purification as well as ADC's instability and tendency for mechanism-based inhibition make enzymatic approaches less attractive (Song *et al.*, 2015). Recently, whole-cell biocatalytic approaches for β-alanine synthesis have been proposed due to the elimination of enzyme processing costs and ADC's increased stability in cells (Li *et al.*, 2018). To further optimize whole-cell approaches, ADC must be engineered to increase its resistance to mechanism-based inhibition. Additionally, a suite of intracellular components, such as co-factor balancing and gene interactions must be addressed in order to optimize whole-cell ADC biosynthesis.

Metabolic role of β-alanine

In *E. coli*, β-alanine and D-pantoic acid are precursors for D-pantothenate (vitamin B5), a precursor for coenzyme A which plays a vital role in lipid synthesis (Cronan 1980). In humans and other mammals, β-alanine is a component in both the dipeptides anserine and carnosine, which are abundantly found in skeletal muscle (Williamson and Brown 1979). In insects, β-alanine plays a role in exoskeleton development (Fukushi 1967), and in plants, it is involved in stress response (Pei *et al.*, 2017).

β-alanine synthesis in microorganisms

In *E. coli*, β-alanine is synthesized primarily through the alpha-decarboxylation of L-aspartate by L-aspartate-alpha-decarboxylase (ADC or PanD) encoded by the *panD* gene (Williamson and Brown, 1979; Cronan 1980). β-alanine can also be yielded from uracil degradation; however, this is a minor pathway for β-alanine production in microorganisms (Cronan 1980). In microorganisms, pantothenate production is preceded by the alpha-decarboxylation pathway for β-alanine synthesis. Pantothenate is synthesized from the condensation of pantoic acid and β-alanine, of which β-alanine is the rate-limiting reactant (Cronan, 1980). Pantothenate is then enzymatically modified into coenzyme A, its metabolically active form (Williamson and Brown, 1979). Therefore, the formation of β-alanine from the alpha-decarboxylation of L-aspartate is an integral part in the formation of coenzyme A in microbes (Williamson and Brown, 1979).

ADC synthesizes β-alanine from L-aspartate

ADC is encoded by the gene *panD*. ADCs can be classified into a bacterial and insect subtype which differ mainly by their cofactor dependence (Wang *et al.*, 2020). Specifically, bacterial ADC utilized by *E. coli*, *Mycobacterium tuberculosis*, and *Bacillus subtilis* is dependent on a pyruvoyl group as a cofactor (Wang *et al.*, 2020). Bacterial ADC is first translated as an inactive pro-protein, which is proteolytically cleaved at the Gly24-Ser25 site to produce the mature, catalytically active form of ADC. Mature ADC consists of an 11 kDa alpha-subunit containing a pyruvoyl group and a 3 kDa β-subunit (Pei *et al.*, 2017, Ramjee *et al.*, 1997). The ADC pro-protein can be cleaved by a separate protein PanZ/PanM or undergo self-cleavage. Stuecker *et al.* (2015) report two classes of pyruvoyl-dependent ADCs: one that must be cleaved by an activator (i.e. PanZ/PanM) and another that can undergo self-cleavage. However, upon analysis of 33 different pyruvoyl-dependent ADCs, Mo *et al.* (2018) propose a third class of pyruvoyl-dependent ADC with an intermediate ability to self-cleave. Under the scheme proposed by Mo *et al.* (2018), Class I pyruvoyl-dependent ADCs require an activator such as PanZ/PanM to cleave the ADC pro-protein, Class II pyuvoyl-dependent ADCs can partially self-cleave, while Class III pyruvoyl-dependent ADCs can completely self-cleave and do not require an activator. For example, *E. coli* ADC relies on PanZ/PanM for cleavage (Pei *et al.*, 2017), *L. plantarum* ADC can only partially self-cleave (Zhang *et al.* 2018), while *B. subtilis* and *Corynebacterium glutamicum* ADCs can fully self-cleave (Pei *et al*. 2017),

Bacterial ADCs belongs to a larger group of pyruvoyl-dependent enzymes, including gram positive histidine decarboxylases (characterized and discovered in *Lactobacillus* 30a (Williamson and Brown, 1979)), *E. coli* phosphatidylserine decarboxylase, *Colistridia* spp. proline reductase (Anton and Kutny, 1987, Williamson and Brown, 1979), *E. coli*, yeast, and rat liver S-adenosylmethionine decarboxylase, *E. coli* phosphatidylserine decarboxylase. Pyruvoyl-dependent enzymes inherently exhibit mechanism-based inactivation, which results in irreversible inactivation of the enzyme (Anton and Kutny 1987). Thus, ADC's tendency for mechanism-based inactivation stands as an obstacle to its efficient application in commercial β-alanine synthesis.

Pyridoxal-5'-Phosphate (PLP)-dependent ADCs are found mainly in eukaryotes such as plants and insects (Yu *et al.*, 2020). One example, the insect ADC found in the red flour beetle, *Tribolium castaneum*, is responsible for providing β-alanine for insect epidermal tanning (Wang *et al.*, 2020). As opposed to pyruvoyl-dependent bacterial ADCs, insect ADC is dependent on pyridoxal-5′-phosphate as a cofactor (Wang *et al.*, 2020). Therefore, insect ADCs are not subject to the same mechanism-based inactivation inherent to bacterial ADCs (Wang *et al.*, 2020). This makes insect ADC an attractive candidate for commercial β-alanine synthesis from L-aspartate.

Metabolic role of β-alanine in mammals

In humans, β-alanine is obtained by the degradation of uracil in the liver and from β-alanine containing-peptides from meats (Lily and Papandreou, 2014). One of β-alanine's main functions in humans is as a precursor molecule to the dipeptide carnosine. Carnosine is a dipeptide formed by the condensation of L-histidine and β-alanine catalyzed by carnosine synthase. Carnosine synthase activity is high in skeletal muscle regions of the brain such as the olfactory bulb, indicating its locations of highest concentration. In skeletal muscles, the imidazole group in the histidine component of carnosine acts as an intracellular cytoplasmic buffer (Lily and Papandreou, 2014). Of the two carnosine precursors, β-alanine is the rate-limiting reactant in carnosine synthesis; therefore, the regulation of intracellular pH in muscle cells is limited by β-alanine concentration. In the blood, serum carnosinase enzymatically hydrolyzes carnosine into its subcomponents, so carnosine is undetectable in the blood(Lily and Papandreou, 2013). Since β-alanine is the rate-limiting reactant in carnosine synthesis, β-alanine supplementation increases intramuscular carnosine concentration. An increase in intramuscular carnosine concentration improves athletic performance and endurance due to the better regulated intracellular pH.

Industrial applications of β-alanine

Due to its role in intracellular pH regulation, particularly during periods of high activity, β-alanine has been used as a supplement to increase endurance in athletes. Additionally, β-alanine has been proposed as an intermediate to industrially-relevant nitrogen-containing compounds such as acrylamide and acrylonitrile (Konst *et al.*, 2009). For example, Konst *et al.* (2009) proposes the conversion of cyanobacterial and yeast cyanophycin-rich biomass grown from agricultural and industrial waste to

β-alanine as an intermediate to valuable nitrogen-containing compounds. β-alanine can also be used as a direct precursor to Nylon-3 (poly-β-alanine), which has applications in cosmetics, water purification, and construction (Steunenberg 2013). The traditional method for Nylon-3 synthesis uses acyrlamide as a precursor but produces unwanted branched byproducts. The β-alanine-based method for Nylon-3 production is instead more efficient but requires a reliable and cost-efficient source of β-alanine. (Song *et al.*, 2015).

Industrial production of β-alanine

Traditionally, β-alanine has been produced through chemical methods, including reacting acrylonitrile with ammonia, introducing β-aminopropionitrile to barium hydroxide in harsh conditions, and reacting acrylic acid with ammonium carbonates and carbon dioxide (Song *et al.*, 2015). These chemical approaches use harsh reactants and produce unwanted by-products, necessitating the development of more environmentally-friendly and efficient methods. More recently, β-alanine has been produced through whole-cell enzymatic systems. Some proposed biological pathways include the conversion of β-aminopropionitrile or aspartic acid to β-alanine in whole-cell systems. For example, Song *et al.* (2015) developed a novel strategy for producing β-alanine in *E. coli*, in which *C. glutamicum* L-asparatate-alpha-decarboxylase (PanD), aspartase (AspA), and phosphoenolpyruvate carboxylase (PPC) genes were overexpressed in *E. coli* W3110.

β-alanine synthesis engineering

β-alanine can be synthesized using ADC and L-aspartate as a substrate. Enzymatic conversion of β-alanine from L-aspartate is more environmentally friendly than the conventional chemical methods.

However, the enzymatic production pathway is limited by ADC mechanism-based inhibition and the high cost of protein extraction and purification. Conversion of L-aspartate to β-alanine through a whole-cell approach is a better alternative to chemical and enzymatic methods since ADC is more stable and shows higher activity *in vivo* (Li *et al.*, 2018)

ADC across organisms

Two subtypes of ADC are present in organisms: the pyruvoyl-dependent ADC in prokaryotes, specifically bacteria, and the PLP-dependent ADC in insects and plants. The majority of studies and genetic engineering on ADC has been focused on the bacterial pyuvate-dependent ADC. Pei *et al*. (2017) studied bacterial ADCs from *E. coli*, *C. glutamicum*, *B. subtilis* and concluded the *B. subtilis* ADC is the best suited for industrial application. *B. subtilis* ADC was more resistant against mechanism-based inactivation, more thermostable, and exhibited higher specific activity than ADCs from *E. coli* and *C. glutamicum* (Pei *et al.*, 2017). Additionally, Zhang *et al.* (2018) demonstrated that *B. subtilis* ADC was more active and stable when compared to *C. glutamicum* and *L. plantarum* ADCs. *B. subtilis* ADC was also able to self-cleave whereas other forms of ADCs (e.g. *E. coli* ADC) require a separate activator, PanZ/PanM, to cleave the ADC pro-protein (Pei *et al.*, 2017). Taken together, *B. subtilis* ADC's ability to self-cleave is advantageous for industrial applications since less gene components need to be engineered into the host for ADC maturation, thereby reducing the metabolic burden on the host.

Due to the inherent mechanism-based inactivation of pyurvoyl-dependent ADCs, more studies have been conducted on insect PLP-dependent ADC to avoid the mechanism-based inactivation. Unlike pyruvoyl-dependent enzymes, PLP-dependent ADC does not need to be cleaved to mature, making it an attractive alternative to pruvoyl-dependent ADCs. Yu *et al.* (2020) genetically engineered *Tribolium castaneum* ADC (TcADC) for industrial β-alanine production. Using random mutagenesis and site-directed saturation mutagenesis, they created a TcADC mutant, (TcPanD-R98H/K305S) that exhibited 2.45 times higher enzymatic activity than the wild type TcADC. Through analysis of their TcPanD-R98H/K305S mutant, Yu *et al.* (2020) propose that the R98H mutation stabilizes the enzyme while the K305S mutation strengthens enzyme-substrate binding. These mutations may serve as a foundation for future optimization of insect ADC. Nevertheless, the majority of protein engineering efforts (e.g. random and/or site-directed mutagenesis) for β-alanine synthesis has been focused on bacterial pyruvoyl-dependent ADC.

Mechanism-based inactivation of pyruvoyl-dependentADC

Mechanism-based inactivation of ADC stands in the way of ADC's industrial application. ADC belongs to a group of enzymes called pyruvoyl-dependent enzymes (Mo *et al.* 2018, Van and Snell 1990) and experiences mechanism-based inactivation common to this group of enzymes (Pei *et al.*, 2017). S-adenosylmethionine decarboxylase is an extensively studied

enzyme that belongs to this group. Studies on S-adenosylmethionine decarboxylase show the mechanism of inactivation for pyruvoyl-dependent enzymes:

A schiff base is formed between the substrate and the pyrovoyl group of the enzyme. The imininium intermediate may be protonated on the substrate or the enzyme, leading to the amination of either the substrate or enzyme. Amination of the substrate leads to production of β-alanine, catalytically regenerating the active enzyme, while amination of the pyruvoyl group leads to the irreversible inactivation of the enzyme. (Pei *et al.*, 2017, Anton and Kutny 1987, Lee and Suh 2004).

Preventing inactivation

The two competing protonation pathways determine whether ADC is inactivated or regenerated for catalysis. Therefore, favoring the desired protonation pathway - the protonation of the substrate over the pyruvoyl group - may prevent the inactivation of ADC (Pei *et al.*, 2017). Pei *et al.* (2017) randomly mutated sequences of the *B. subtilis* ADC and screened for higher enzymatic activity and catalytic stability in ADC variants. From 4000 variants, they found two variants which exhibited 18–22% higher enzymatic activity and 29–64% higher catalytic stability than wild type ADC. Analyzing their ADC variants, Pei *et al.* (2017) found that mutations in the C-terminus, specifically L127, the last amino acid in B. subtilis ADC increased its catalytic stability. Additionally, amino acid sequence alignments between 16 ADC genes showed the most variation in ADC's C-terminus loop (Pei *et al*., 2017, Lee and

Suh 2004). These findings suggest future engineering of this location may improve ADC's resistance to mechanism-based inactivation. Indeed, Qian *et al.* (2020) demonstrated a C-terminal deletion in *B. subtilis* ADC favored the desired protonation pathway.

Zhang *et al.* (2018) performed site-directed mutagenesis on *B. subtilis* ADC and found their Glu56Ser ADC mutant exhibited 1.6-fold higher catalytic activity, increased thermostability, and decreased mechanism-based inactivation. Their Glu56Ser *B. subtilis* ADC mutant was heterologously expressed in E. coli BL21 which produced 215.3 g/L β-alanine, the highest β-alanine biosynthesis yield reported so far.
Qian *et al.* (2020) propose two factors affect the mechanism-based inactivation of pyruvoyl-dependent ADCs. A short distance between the proton donor Tyr58 and the pyruvoyl group results in a higher likelihood of the protonation of the enzyme, leading to the irreversible inactivation of the enzyme. The remedy for this problem is increasing the protonation distance between the proton donor and the pyruvoyl group - doing so will increase the energy barrier needed to protonate the enzyme, thereby increasing the likelihood that the correct protonation pathway is followed. Second, the structural flexibility of ADC may affect which protonation pathway occurs. Excessive flexibility of ADC fosters the incorrect enzyme configuration for protonation of the substrate. Rather, a more stable configuration may favor the desired protonation pathway. Through site-directed saturation mutation and c-terminus deletion, Qian *et*

al. produced a *B. subtilis* ADC mutant that remedies both of these problems; their mutant ADC Q5 exhibited a 3.48-times higher half-life and a 2.52 times higher total turnover number.

Whole-cell approaches

Li *et al.* (2018) employ a whole-cell catalytic approach using *C. glutamicum* ADC expressed in E. coli BL21 (BL21(DE3)/pET28a-panDC)yielding 24.8 g/L β-alanine from 40 g/L L-aspartate (92.6% yield). Their whole-cell approach showed higher β-alanine production than that of the crude extracts. Although the cell membrane limits import of starting material and export of accumulated product. Li *et al.* (2018) propose that their whole-cell approach produces more β-alanine because the cell membrane prevents L-aspartate substrate inhibition of ADC that occurs when producing β-alanine with crude extract.

Wang *et al.* (2020) employ a novel two-enzyme whole-cell system to convert L-aspartate to β-alanine. Wang *et al.* rely on *B. subtilis* ADC and *T. castaneum* ADC, which represent the two subtypes of the ADC enzyme - *B. subtilis* ADC relies on a pyruvoyl group as a cofactor while *T. castaneum* ADC relies on pyridoxal-5′-phosphate as a cofactor. This study co-expressed *B. subtilis* ADC and *T. castaneum* ADC in *E. coli* and displayed a 92.4% conversion rate from L-aspartate to β-alanine. Additionally, Wang *et al.* (2020) developed a "one-pot" three-enzyme system to convert fumaric acid to β-alanine. Starting with fumaric acid, Wang *et al.* use *E. coli* aspartase-catalyzed ammonization to convert fumaric acid to L-asparte and *B. subtilis* ADC and *T. castaneum* ADC to convert L-aspartate to β alanine. Their one-pot method yielded a 90% conversion rate from fumaric acid to β-alanine.

De novo synthesis of β-alanine

Some whole-cell methods have employed a fed-batch approach to produce β-alanine from L-aspartate or fumarate. Other β-alanine production engineering focuses instead on synthesizing β-alanine *de novo* from glucose. For example, Song *et al.* (2015) achieved a yield of 0.135 g β-alanine/g glucose at a productivity of 0.828 g/L/h by overexpressing *Corynebacterium glutamicum* ADC, native asparatase, and phosphoenolpyruvate carboxylase. Similarly, Piao *et al.* (2019) increased their β-alanine yield from glucose by optimizing L-aspartate production, specifically increasing L-aspartate aminotransferase and ADC expression in E. coli.

TCA pathways for de novo β-alanine synthesis

Increasing the supply of L-aspartate, the direct precursor of β-alanine, should improve the theoretical yield of β-alanine in a whole-cell system. L-aspartate can be produced from fumarate or oxaloacetate through a number of pathways. Specifically, phosphoenolpyruvate (PEP) is converted to fumarate, an L-aspartate precursor, through the oxidative branch of the TCA cycle. This pathway synthesizes L-aspartate - and subsequently β-alanine - at a theoretical yield of 1 mol β-alanine/mol glucose (Piao *et al.* 2019). 1.33 mol L-aspartate/mol glucose can theoretically be synthesized from supplementing the oxidative branch of the TCA cycle with the glyoxylate cycle, which replenishes TCA cycle intermediates (Piao *et al.* 2019). Song *et al.* (2015) were the first to develop de novo synthesis of β-alanine from glucose by augmenting L-aspartate synthesis via the oxidative arm of the TCA cycle. By replacing the native promoter

for *aspA*, an aspartate ammonia-lyase-encoding gene, with the strong *trc* promoter, they were able to increase fumarate conversion to L-aspartate. Additional expression of *Corynebacterium glutamicum* ADC and *ppc*, a phosphoenolpyruvate carboxylase-encoding gene, increased their engineered strain's β-alanine productivity to 0.828 g/L/h. This yield is relatively low due to the loss of CO2 through the oxidative branch of the TCA cycle.

Conversely, Piao *et al.* (2019) proposes the most efficient L-aspartate synthesis pathway is the reductive branch of the TCA cycle, which generates oxaloacetate from PEP. This pathway has the highest L-aspartate theoretical yield at 2 mol L-aspartate/mol glucose. The reductive branch of the TCA cycle conserves carbon, whereas the oxidative TCA pathway and glyoxylate cycle lose carbon through CO2. The reductive TCA branch is the shortest pathway from PEP to L-aspartate; therefore, utilizing it for β-alanine synthesis may bypass complicated regulation associated with other pathways (Piao *et al.* 2019). Piao *et al.* (2019) overexpressed *C. glutamicum ppc* to increase the conversion of PEP to oxaloacetate and *Bacillus subtilis* ADC to drive metabolic flux to L-aspartate and β-alanine synthesis pathways. Further, to reduce competitive oxaloacetate- and L-aspartate-consuming pathways, Piao *et al.* deleted *mdh*, a malate dehydrogenase-encoding gene, and *aspA* (aspartate ammonia-lyase catalyzes the reversible conversion between fumarate and L-aspartate (Piao *et al.* 2019)). With these genetic modifications, their highest β-alanine yield was 1.52 mol β-alanine/mol glucose, equivalent to 76% of the theoretical yield.

Some alternative methods for biosynthesizing β-alanine include extracellularly expressing ADC for β-alanine conversion from L-aspartate. To do this, Feng *et al.* (2019) expressed a *Bacillus tequilensis* ADC tagged with signal peptides for transmembrane export. Additionally, they co-expresssed the ADC with *T. fusca* cutinase, which catalyzes phospholipid hydrolysis, to increase non-specific release of intracellular proteins. The export of overexpressed ADC relieves the cell of the metabolic burden caused by exogenous protein accumulation in the cell, theoretically increasing the productivity of the cell. To prevent the substrate inhibition of ADC by L-aspartate at high concentrations, Feng *et al.* (2019) utilized a continuous L-aspartate feeding regimen. With their novel approach, Feng *et al.* demonstrated extracellular expression of ADC exhibited 1.4-fold improvement in β-alanine production than intracellular ADC expression.

Perspective

The majority of the work on augmenting β-alanine biosynthesis has been concentrated on optimizing L-aspartate conversion to β-alanine by pyruvoyl-dependent ADCs. Pyruvoyl-dependent ADCs are more well-studied and more prevalent in microorganisms. However, they are limited by mechanism-based inhibition and the need for activation by cleavage; thus, they require continued improvement through protein engineering. There have been comparatively less studies on PLP-dependent ADCs, which do not exhibit substrate inhibition and do not need to be cleaved to fully mature. PLP-dependent ADC may be a promising enzyme for β-alanine biosynthesis but has not been widely used for this purpose. Strategies to improve both subtypes of ADCs have mainly been random mutagenesis and site-directed saturation mutagenesis, which continue to be the most efficient

methods for protein optimization. To date, the highest β-alanine yield was obtained by Zhang *et al.* (2018) who optimized a *B. subtilis* pyruvoyl-dependent ADC through site-directed mutagenesis. Moving forward, continued protein engineering of pyurvoyl-dependent ADCs and synthesis of β-alanine through a whole-cell, *de novo* approach may result in the most cost-efficient and highest β-alanine yield.

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